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Health and Environmental Effects Profile for Direct Blue 6

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16. ABSTRACT

The Health and Environmental Effects Profile for Direct Blue 6 was prepared by the Office of Health and Environmental Assessment, Environmental Criteria and Assessment Office, Cincinnati, OH for the Office of Solid Waste and Emergency Response to support listings of hazardous constituents of a wide range of waste streams under Section 3001 of the Resource Conservation and Recovery Act (RCRA) and to provide health-related limits for emergency actions under Section 101 of the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA). Both published literature and information obtained from Agency program office files were evaluated as they pertained to potential human health, aquatic life and environmental effects of hazardous waste constituents.

Quantitative estimates have been presented provided sufficient data are available. Direct Blue 6 has been evaluated as a carcinogen. The human carcinogen potency factor (q_1^*) for Direct Blue 6 is 8.1 $(mg/kg/day)^{-1}$ for oral exposure.

The Reportable Quantity (RQ) value of 1, 10, 100, 1000 or 5000 pounds is used to determine the quantity of a hazardous substance for which notification is required in the event of a release as specified by CERCLA based on chronic toxicity. The RQ value for Direct Blue 6 is 100.

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HEALTH AND ENVIRONMENTAL EFFECTS PROFILE FOR DIRECT BLUE 6

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PREFACE

Health and Environmental Effects Profiles (HEEPs) are prepared for the Office of Solid Waste and Emergency Response by the Office of Health and Environmental Assessment. The HEEPs are intended to support listings of hazardous constituents of a wide range of waste streams under Section 3001 of the Resource Conservation and Recovery Act (RCRA), as well as to provide health-related limits for emergency actions under Section 101 of the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA). Both published literature and information obtained from Agency program office files are evaluated as they pertain to potential human health, aquatic life and environmental effects of hazardous waste constituents. The literature searched and the dates of the searches are included in the section titled "Appendix: Literature Searched." The literature search material is current through November, 1985.

Quantitative estimates are presented provided sufficient data are available. For systemic toxicants, these include Reference doses (RfDs) for chronic exposures. An RfD (formerly known as the ADI) is defined as the amount of a chemical to which humans can be exposed on a daily basis over an extended period of time (usually a lifetime) without suffering a deleterious effect. In the case of suspected carcinogens, RfDs are not estimated in this document series. Instead, a carcinogenic potency factor or q_1^* is provided. These potency estimates are derived for both oral and inhalation exposures where possible. In addition, unit risk estimates for air and drinking water are presented based on inhalation and oral data, respectively.

Reportable quantities (RQs) based on both chronic toxicity and carcinogenicity are derived. The RQ is used to determine the quantity of a hazardous substance for which notification is required in the event of a release as specified under CERCLA. These two RQs (chronic toxicity and carcinogenicity) represent two of six scores developed (the remaining four reflect ignitability, reactivity, aquatic toxicity and acute mammalian toxicity).

The first draft of this document was prepared by Syracuse Research Corporation under EPA Contract No. 68-03-3228. The document was subsequently revised after reviews by staff within the Office of Health and Environmental Assessment: Carcinogen Assessment Group, Reproductive Effects Assessment Group, Exposure Assessment Group, and the Environmental Criteria and Assessment Office in Cincinnati.

The HEEPs will become part of the EPA RCRA and CERCLA dockets.

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The initial draft of this report was prepared by Syracuse Research Corporation under Contract No. 68-03-3228 for U.S. EPA's Office of Health and Environmental Assessment, Environmental Criteria and Assessment Office, Cincinnati, OH. Dr. Christopher DeRosa was the Technical Project Monitor and Helen Ball was the Project Officer. The final documents in this series were prepared for the Office of Solid Waste, Washington, DC.

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EXECUTIVE SUMMARY

Direct Blue 6 is a common name for a commercial azo dye, which is produced, in part, by the diazotization of benzidine (The Society of Dyers and Colourists, 1971). It is soluble in water and forms anions by dissociation (Kuehni et al., 1979). It can be used to dye, stain or print cellulose, silk, leather, paper, wool, nylon and biological materials (IARC, 1982a). Eight U.S. manufacturers produced 0.288 million pounds of this dye in 1972 (USITC, 1974), but only one U.S. manufacturer reported commercial production of Direct Blue 6 in 1982 (USITC, 1983). One U.S. company currently markets Direct Blue under two tradenames (AATCC, 1985).

Pertinent data regarding the environmental fate of Direct Blue 6 could not be located in the available literature as cited in the Appendix. If released to water, Direct Blue 6 may be susceptible to significant adsorption because dyestuffs, by their substantive nature (ability to be exhaustively deposited to fibers from aqueous baths), are likely to be adsorbed onto silts and sediment of rivers and lakes (Brown and Laboureur, 1983). Removal of adsorbed Direct Blue 6 may occur by anaerobic biodegradation since a number of azo dyes have been found to be substantially biodegraded under anaerobic conditions (Brown and Laboureur, 1983). In general, direct dyes are expected to be relatively stable to direct photolysis in natural waters (Porter, 1973), but humic material in natural water has been found to strongly accelerate the photodecomposition of azo dyes (Haag and Mill, 1985). Therefore, indirect photolysis may also be significant. Volatilization and bioconcentration are not expected to be significant. It appears that indirect photolysis, microbial degradation and adsorption are the important fate processes in water. If released to the atmosphere, Direct

Blue 6 will probably be present as particulate matter and dusts and may be removed from the atmosphere by wet and dry deposition. If released to soil, Direct Blue 6 may not leach significantly since dyestuffs are susceptible to significant adsorption. It is susceptible to anaerobic biodegradation in soil (Brown and Labourer, 1983). Photodegradation in soil may not be significant beyond the surficial layer. The role of hydrolysis and chemical reduction in soil remains unknown.

Occupational exposure to Direct Blue 6 has occurred, and probably still occurs, during its production and use in dyeing (IARC, 1982a). Wastewater effluents are probably the major source of release to the environment. Ambient monitoring data could not be located in the available literature.

Little et al. (1974) reported that the 96-hour LC_{50} of Direct Blue 6 to fathead minnows, Pimephales promelas, was >180 mg/ℓ. No other information concerning aquatic toxicity of this compound was available.

Data regarding the amount or rate of absorption of Direct Blue 6 were not available. The distribution of Direct Blue 6 was studied by Beaudoin and Pickering (1960), who injected pregnant rats intraperitoneally with 140 mg/kg Direct Blue 6 on day 8 of gestation and detected Direct Blue 6 as granules in the cytoplasm of macrophage cells from the lungs, liver, uterus, ovaries, abdominal lymph nodes and kidneys.

Kennelly et al. (1984) identified N-(de-oxyguanosin-8-yl)-N'-acetylbenzidine and N-(deoxyguanosin-8-yl) benzidine as DNA adducts in the liver of rats injected intraperitoneally with ^{3}H-ring-Direct Blue 6. Direct Blue 6 is metabolized to benzidine and acetylbenzidine. In addition to reduction to benzidine, Direct Blue 6 may undergo hepatic azoreduction producing a species which may also react with DNA. In vitro experiments with S-9 and isolated rat hepatocytes indicated that the production of

benzidine is catalyzed by microsomal enzymes, while the subsequent acetylation of benzidine is catalyzed by cytosolic enzymes (Bos et al., 1984). Benzidine and monoacetylbenzidine have been detected in the urine of workers exposed to benzidine-based dyes (NIOSH, 1980a) and rats, mice and monkeys exposed orally to Direct Blue 6 (NCI, 1978; Rinde and Troll, 1975).

Direct Blue 6 has been demonstrated to be a potent carcinogen in the rat. Rats exposed to dietary concentrations of 190, 375, 750, 1500 or 3000 ppm for only 13 weeks had significantly increased incidences of liver tumors (hepatocarcinomas and neoplastic nodules) at $\geq$1500 ppm (NCI, 1978). Liver tumors were not observed in controls or in lower dose groups. The time to first tumor was 4-5 weeks in treated rats compared with 78 weeks in the historic controls, indicating that Direct Blue 6 is a remarkably potent carcinogen. No increased incidences of tumors were observed in mice at dietary levels $\geq$750 ppm (lowest level tested). Niitsu (1973) gave rats 500 mg/ℓ Direct Blue 6 in drinking water for 14 months and found that only one rat had a glandular tumor of the outer ear. No controls were maintained in this experiment.

There are numerous case reports available (IARC 1982a,b,c) suggesting that Direct Blue 6 is carcinogenic in humans. Yoshida and Miyakawa (1973) and Yoshida et al. (1971) observed 17 cases of bladder cancer in a hospital-based case-control study of 200 workers occupationally exposed to benzidine-based dyes. An incidence of only 2/148 was observed in controls. Seven of the cases were kimono painters who held brushes and spatulas in their mouths while painting, thus ingesting the dyes. IARC (1982a,b) concluded that exposure to benzidine-based dyes was strongly associated with the occurrence of bladder cancer in humans.

The mutagenicity of Direct Blue 6 was assessed in the Salmonella reverse mutation assay. Positive results were obtained in tester strains TA98 and TA1538 only in the presence of a reduction system (Prival et al., 1984; Reid et al., 1984). Direct Blue 6 is reduced to the carcinogen benzidine, which has been reported as mutagenic in Salmonella tester strains TA98 and TA1538. In cultured mammalian cells, benzidine produces sister chromatid exchanges, unscheduled DNA synthesis, and DNA strand breaks (Grady et al., 1986; IARC, 1982).

No data concerning the teratogenic effects of Direct Blue 6 after oral or inhalation exposure were located; however, several positive studies by the subcutaneous or intraperitoneal route were found. Dose-related increased incidences of resorptions and fetal abnormalities were observed in female rats given 50-200 mg/kg of Direct Blue 6 subcutaneously during pregnancy (Lloyd and beck, 1966; Kernis, 1971). A limitation of the Lloyd and Beck (1966) study was that no controls were maintained. Controls were maintained in Kernis (1971); however, no maternal toxicity data were reported. Beaudoin (1968) injected pregnant rats intraperitoneally with 0, 70, 140 or 200 mg/kg Direct Blue 6 and observed dose-related increased resorptions and malformed fetuses (anophthalmia, hydrocephalus at all doses); however, maternal lethality was observed at the two highest doses.

Pertinent data regarding the reproductive effects of Direct Blue 6 could not be located in the available literature as cited in the Appendix.

In the 13-week dietary carcinogenicity study by NCI (1978), dose-related decreased mean body weight gain of rats occurred at $\geq$1500 ppm. All 3000 ppm rats and 1/10 males at 1500 ppm died. Treatment-related nonneoplastic lesions in rats included biliary hyperplasia in males at $\geq$750 ppm, nodular regeneration and portal fibrosis in males and females at 1500 ppm, lymphoid

depletion of the thymus and spleen and myeloid depletion of the bone marrow at 3000 ppm. None of these lesions occurred in controls. Mice treated with 12,500 ppm (highest dose) had only slightly reduced body weight gain, and all mice at $\geq$750 (lowest dose) survived. Nonneoplastic lesions were hemosiderosis of kidney and pigment deposition in the liver at 12,500 ppm and hemosiderosis of the spleen at $\geq$1500 ppm.

A human q_1^* of 8.1 $(mg/kg/day)^{-1}$ was derived based on the incidence of hepatocellular carcinoma or neoplastic nodule in male rats after dietary exposure to Direct Blue 6 in the NCI (1978) study. The concentrations in water associated with increased lifetime risk at levels of 10^{-5}, 10^{-6} and 10^{-7} are 4.3×10^{-5}, 4.3×10^{-6} and 4.3×10^{-7} mg/ℓ, respectively, assuming that a 70 kg man drinks 2 ℓ water/day. An F factor of 92 $(mg/kg/day)^{-1}$ was also calculated from the data for liver tumors in male rats.

A consideration of metabolism and related factors is important in that formulations of Direct Blue 6 apparently have varying amounts of residual benzidine as a contaminant and that mammals including monkeys have been shown to metabolize Direct Blue 6 to benzidine and acetalbenzidine to some degree. The presence of benzidine in the urine of workers exposed to benzidine-based dyes together with significant incidences of bladder cancer demonstrates an important correlation.

The animal evidence is limited, there being liver tumors induced in rats exposed by dietary administration but not in mice. Other experiments show that Direct Blue 6 is metabolized in part to benzidine and acetalbenzidine, albeit a small amount. By other carcinogen assessments benzidine is an EPA Group A compound, a known human carcinogen. The animal evidence together with knowledge that some Direct Blue 6 is metabolized to benzidine and the

knowledge that Direct Blue 6 formulations undoubtedly contain residual amounts of benzidine, elevate the weight of evidence for Direct Blue 6 to a Group A categorization.

In a collaborative manner, a consideration of the available epidemiologic data for benzidine-based dyes with the clear findings of human bladder cancer response, together with presence of benzidine in the urine of exposed individuals, is supportive of a Group A ranking for benzidine-based dyes of which Direct Blue 6 is one. An F factor of 92 $(mg/kg/day)^{-1}$ places Direct Blue in potency Group 2 which, along with the A classification, gives Direct Blue a HIGH hazard ranking under CERCLA.

TABLE OF CONTENTS

		Page
1.	INTRODUCTION	1
1.1.	STRUCTURE AND CAS NUMBER	1
1.2.	PHYSICAL AND CHEMICAL PROPERTIES	1
1.3.	PRODUCTION DATA	2
1.4.	USE DATA	4
1.5.	SUMMARY	4
2.	ENVIRONMENTAL FATE AND TRANSPORT PROCESSES	5
2.1.	WATER	5
2.1.1.	Hydrolysis	5
2.1.2.	Oxidation/Reduction	5
2.1.3.	Photolysis	5
2.1.4.	Microbial Degradation	6
2.1.5.	Volatilization	6
2.1.6.	Adsorption	6
2.1.7.	Bioconcentration	7
2.2.	AIR	7
2.3.	SOIL	7
2.4.	SUMMARY	8
3.	EXPOSURE	10
3.1.	WATER	10
3.2.	FOOD	11
3.3.	INHALATION	11
3.4.	DERMAL	11
3.5.	SUMMARY	11
4.	PHARMACOKINETCS	12
4.1.	ABSORPTION	12
4.2.	DISTRIBUTION	12
4.3.	METABOLISM	12
4.4.	EXCRETION	14
4.5.	SUMMARY	14
5.	EFFECTS	16
5.1.	CARCINOGENICITY	16
5.1.1	Animal Studies	16
5.1.2.	Human Studies	19

TABLE OF CONTENTS (cont.)

		Page
5.2.	MUTAGENICITY	20
5.3.	TERATOGENICITY	22
5.4.	OTHER REPRODUCTIVE EFFECTS	23
5.5.	CHRONIC AND SUBCHRONIC TOXICITY	23
5.6.	OTHER RELEVANT INFORMATION	25
5.7.	SUMMARY	25
6.	AQUATIC TOXICITY	29
6.1.	ACUTE	29
6.2.	CHRONIC	29
6.3.	PLANTS	29
6.4.	RESIDUES	29
6.5.	SUMMARY	29
7.	EXISTING GUIDELINES AND STANDARDS	30
7.1.	HUMAN	30
7.2.	AQUATIC	30
8.	RISK ASSESSMENT	31
9.	REPORTABLE QUANTITIES	36
9.1.	REPORTABLE QUANTITY (RQ) RANKING BASED ON CHRONIC TOXICITY	36
9.2.	WEIGHT OF EVIDENCE AND POTENCY FACTOR ($F=1/ED_{10}$) FOR CARCINOGENICITY	39
10.	REFERENCES	45
APPENDIX:	LITERATURE SEARCHED	53

LIST OF TABLES

No.	Title	Page
1-1	Direct Blue 6 Production Data for 1977.	3
5-1	Incidence of Liver Tumors in Fischer 344 Rats Given 190-3000 ppm of Direct Blue 6 (no benzidine contamination) in the Diet for 91 Days and Observed for an Additional 2 Days.	17
5-2	Mutagenicity Testing of Direct Blue 6	21
8-1	Derivation of a q_1^* for Direct Blue 6	35
9-1	Oral Toxicity Summary for Benzidine-Free Direct Blue 6 Administered by Diet (10 Animals/Group)	37
9-2	Oral Composite Scores for Direct Blue 6 in Fischer 344 Rats .	38
9-3	Direct Blue 6: Minimum Effective Dose (MED) and Reportable Quantity (RQ)	40
9-4	Derivation of Potency Factor (F) for Direct Blue 6.	43

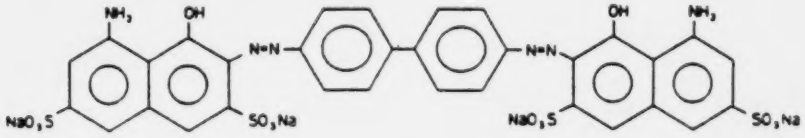
LIST OF ABBREVIATIONS

BCF	Bioconcentration factor
bw	Body weight
CAS	Chemical Abstract Service
CS	Composite score
FMN	Flavinmononucleatide
K_{ow}	Octanol/water partition coefficient
LC_{50}	Concentration lethal to 50% of recipients
LD_{50}	Dose lethal to 50% of recipients
MED	Minimum effect dose
MTD	Maximum tolerated dose
ppm	Parts per million
RQ	Reportable quantity
RV_d	Dose-rating value
RV_e	Effect-rating value
TWA	Time-weighted average
UV	Ultraviolet

1. INTRODUCTION

1.1. STRUCTURE AND CAS NUMBER

Direct Blue 6 is a common name for the compound known by the IUPAC system of chemical nomenclature as tetrasodium 3,3'-[4,4-bi-phenylenebis-(azo)]bis(5-amino-4-hydroxy)-2,7-naphthalenedisulfonate. The current CAS designated name for Direct Blue 6 is 2,7-naphthalenedisulfonic acid, 3,3'-[(1,1'-biphenyl)-4,4diylbis(azo)]-bis(5-amino-4-hydroxy)-, tetra sodium salt. Direct Blue 6 is currently marketed in the United States under the tradenames Amidine 2B Concentrate and Amidine 2BNB Concentrate (AATCC, 1985). The structure, molecular weight, empirical formula and CAS number for Direct Blue 6 are as follows:



Molecular weight: 936.8

Empirical formula: $C_{32}H_{20}N_6Na_4O_{14}S_4$

CAS Number: 2602-46-2

1.2. PHYSICAL AND CHEMICAL PROPERTIES

Direct Blue 6 is one of many dyes belonging to the azo dye grouping. Most of the direct azo dyes are produced from benzidine or benzidine congeners. Direct Blue 6 is a blue-violet solid (IARC, 1982a). It is soluble in water, forming a blue to navy blue solution, slightly soluble in ethanol and ethylene glycol monoethyl ether and insoluble in most other organic solvents (The Society of Dyers and Colourists, 1971). All direct dyes are water soluble and form anions by dissociation (Kuehni et al., 1979). In aqueous solution, the molecules of direct dyestuffs are linked by hydrogen bonds, forming larger agglomerates or collodial solutions, which

reduces their solubility and promotes disposition on cellulosic fibers. The structure of these agglomerates allows firm linkage to the cellulose molecule by multiple hydrogen bonds (Kuehni et al., 1979).

Physical properties such as melting point, boiling point, vapor pressure, density and log K_{ow} could not be located in the available literature as cited in the Appendix. Lack of appropriate fragment values precluded the computer calculation of log K_{ow} (U.S. EPA, 1986a). The air conversion factor for Direct Blue 6 is 1 ppm = 0.0261 mg/m³ (IARC, 1982a).

The manufacture and testing of Direct Blue 6 do not conform to rigid chemical specifications, and its composition may vary in order to meet shade and intensity requirements of each customer (IARC, 1982a). The residual benzidine content of two U.S.-produced Direct Blue 6 samples were 4 and 12 mg/kg (IARC, 1982a).

1.3. PRODUCTION DATA

Direct Blue 6 was first produced in commercial quantities in the United States in 1914 (IARC, 1982a). It is produced by coupling diazotized benzidine with 2 mol of H-acid (8-amino-1-naphthol-3,6-disulfonic acid) under alkaline conditions (The Society of Dyers and Colourists, 1971).

In 1972, eight U.S. companies produced 0.288 million pounds of Direct Blue 6 (USITC, 1974); this is the most recent production figure available from the U.S. International Trade Commission (formerly the U.S. Tariff Commission). Sales in 1973 were reported to be 0.327 million pounds (USITC, 1975). 1982 is the last year the USITC reported individual production of Direct Blue 6, with Mobay Chemical listed as the only manufacturer (USITC, 1983). John Campbell & Co. currently markets Direct Blue 6 in the United States (AATCC, 1985). The production data available from the public portion of the U.S. EPA TSCA Production File for 1977 is listed in Table 1-1. Data concerning current U.S. production were not available.

TABLE 1-1

Direct Blue 6 Production Data for 1977*

Producer/Location	Type of Producer	Production Range (thousand pounds)
Fabricolor Corp. Paterson, NJ	manufacturer	10-100
GAF Corp. Rensselaer, NY	manufacturer	10-100
Ugine Kuhlmann of America Paramus, NJ	importer	confidential
L & R Dyestuffs Corp Clifton, NJ	importer	1-10
Confidential	confidential	<1000 pounds

*Source: U.S. EPA, 1977

Import of Direct Blue 6 through principal U.S. customs districts in 1978, the last reported year, was 4409 pounds. (USITC, 1979).

1.4. USE DATA

Direct Blue 6 is a dye that can be used to 1) dye cellulose, silk, leather and paper; 2) stain silk, wool, nylon and biological materials; 3) print cellulose fabrics and 4) produce aqueous writing inks (IARC, 1982a). It has been used in hair dyes (Feinland et al., 1980).

1.5. SUMMARY

Direct Blue 6 is a common name for a commercial azo dye, which is produced, in part, by the diazotization of benzidine (The Society of Dyers and Colourists, 1971). It is soluble in water and forms anion by dissociation (Kuehni et al., 1979). It can be used to dye, stain or print cellulose, silk, leather, paper, wool, nylon and biological materials (IARC, 1982a). Eight U.S. manufacturers produced 0.288 million pounds of this dye in 1972 (USITC, 1974), but only one U.S. manufacturer reported commercial production of Direct Blue 6 in 1982 (USITC, 1983). One U.S. company currently markets Direct Blue under two tradenames (AATCC, 1985).

2. ENVIRONMENTAL FATE AND TRANSPORT PROCESSES

2.1. WATER

2.1.1. Hydrolysis. In the absence of experimental hydrolysis data on the dissociation constant of the free acid, it is difficult to speculate on the hdyrolytic fate of this compound. Additional relevant data could not be located in the available literature as cited in the Appendix.

2.1.2. Oxidation/Reduction. Takemura et al. (1965) have reported that bubbling of H_2S through a pure azo-dye solution yields aromatic amines and have suggested that azo dyes in wastewater may be reduced at the azo linkage (by H_2S or SO_2 in the water) to form aromatic amines such as benzidine or naphthylamine from which the dye was produced. The detection of aromatic amines in Sumida River, an especially polluted river in Japan, was speculated to be due partly to this reduction of discharged azo dyes by H_2S or SO_2 present in the river (Takemura et al., 1965).

2.1.3. Photolysis. Porter (1973) examined the photodegradation rate in aqueous solution of eight direct dyes and 12 other azo dyes in artificial light, and one direct dye in natural sunlight. Based on the experimental results, the author concluded that direct dyes are relatively stable to direct photolysis in natural waters. Although Direct Blue 6 was not one of the dyes studied, its structure is similar to several dyes that were tested.

Haag and Mill (1985) examined the aqueous photodegradation rate of 15 azo dyes by simulated sunlight or natural sunlight. Potentially significant direct photolysis was observed for some dyes; however, humic material in natural water were found to strongly accelerate the photodecomposition rate of all the dyes. Therefore, indirect photolysis may be an important removal mechanism for azo dyes in the aquatic environment.

2.1.4. Microbial Degradation. Pertinent data regarding the microbial degradation of Direct Blue 6 could not be located in the available literature as cited in the Appendix. Yoshida et al. (1981) found that the benzidine-derived dye Direct Black 38 was reduced by natural river water or soil inocula; however, reduction did not occur when the river water or soil was sterilized, which is indicative of microbial degradation.

Brown and Laboureur (1983) reported that a number of azo dyes were substantially biodegraded under anaerobic test conditions using sludge inocula and suggested that the breakdown of dyestuffs in the environment may be initiated under anaerobic conditions.

2.1.5. Volatilization. Experimental data could not be located in the available literature; however, Direct Blue 6 is soluble in water (The Society of Dyers and Colourists, 1971) and is expected to have a relatively low vapor pressure, based on its chemical structure, which would predict a relatively small Henry's Law Constant. Therefore, volatilization from water is not expected to be significant in the environment.

2.1.6. Adsorption. Pertinent data regarding the adsorption of Direct Blue 6 could not be located in the available literature as cited in the Appendix; however, dyestuffs by their substantive nature (ability to be exhaustively deposited to fibers from aqueous baths) are likely to be adsorbed onto both sewage sludge, silts and sediments of rivers and lakes (Brown and Laboureur, 1983). In conventional biological waste treatment systems, soluble dye removal usually occurs when the dye is adsorbed onto sludge (Porter, 1973). Sewage works sludge is usually treated by anaerobic digestion and/or landfilling. Removal of the dye from the sludge may therefore occur by anaerobic biodegradation. Similar anaerobic conditions exist in many lakes and rivers with respect to adsorbed material on silts

and sediments, and, therefore, adsorbed dyes may be subject to anaerobic biodegradation in the environment (Brown and Laboureur, 1983).

2.1.7. Bioconcentration. Pertinent data regarding the bioconcentration of Direct Blue 6 could not be located in the available literature as cited in the Appendix. Since Direct Blue 6 is water soluble (The Society of Dyers and Colourists, 1971), bioconcentration in aquatic organisms is not expected to be significant.

2.2. AIR

Direct Blue 6 in the atmosphere is most likely to be present as particulate matter and dusts, especially from atmospheric effluents resulting from production or use. These particulate matter and dusts are subject to wet and dry deposition. Since the compound contains amine substituents, it may also undergo heterogeneous oxidation reactions with OH radical present in the atmosphere; however, none of the atmospheric fate processes of this compound has been studied.

2.3. SOIL

Pertinent data regarding the environmental or chemical fate of Direct Blue 6 in the soil could not be located in the available literature as cited in the Appendix. Of the abiotic processes, the three that may influence the fate of Direct Blue 6 in soil are hydrolysis, reduction and photolysis. Because of light attenuation and scattering, photolysis may not be significant beyond the surficial layer of soil. The role of hydrolysis and reduction in soils has not been studied, although reduction at the azo group with the formation of amines may occur under reducing soil conditions. A number of azo dyes have been shown to be susceptible to anaerobic biodegradation (Brown and Laboureur, 1983), and the benzidine-derived dye Direct Black 38 has been shown to be reduced with soil inocula (Yoshida et al., 1981).

Since Direct Blue 6 is water soluble (The Society of Dyers and Colourists, 1971), leaching in soil might be expected; however, the substantive nature of dyestuffs (Brown and Laboureur, 1983), including the direct dyes (Kuehni et al., 1979), indicates that significant adsorption to soil may occur. Therefore, significant leaching in soil may not occur.

2.4. SUMMARY

Pertinent data regarding the environmental fate of Direct Blue 6 could not be located in the available literature as cited in the Appendix. If released to water, Direct Blue 6 may be susceptible to significant adsorption because dyestuffs, by their substantive nature (ability to be exhaustively deposited to fibers from aqueous baths), are likely to be adsorbed onto silts and sediment of rivers and lakes (Brown and Laboureur, 1983). Removal of adsorbed Direct Blue 6 may occur by anaerobic biodegradation since a number of azo dyes have been found to be substantially biodegraded under anaerobic conditions (Brown and Laboureur, 1983). In general, direct dyes are expected to be relatively stable to direct photolysis in natural waters (Porter, 1973), but humic material in natural water has been found to strongly accelerate the photodecomposition of azo dyes (Haag and Mill, 1985). Therefore, indirect photolysis may also be significant. Volatilization and bioconcentration are not expected to be significant. It appears that indirect photolysis, microbial degradation and adsorption are the important fate processes in water. If released to the atmosphere, Direct Blue 6 will probably be present on particulate matter and dusts and may be removed from the atmosphere by wet and dry deposition. If released to soil, Direct Blue 6 may not leach significantly since dyestuffs are susceptible to significant adsorption. It may be susceptible to anaerobic biodegradation

in soil (Brown and Labourer, 1983). Photodegradation in soil may not be significant beyond the surficial layer. The role of hydrolysis and chemical reduction in soil remains unknown.

3. EXPOSURE

Direct Blue 6 has not been reported to occur in nature (IARC, 1982a); therefore, exposure from natural sources is not expected to occur. Occupational exposure to Direct Blue 6 has occurred (and probably still does) during its production and its use for the dyeing of textiles, leather and paper (IARC, 1982a). Likely routes of exposure are inhalation of particulates and dermal exposure. A National Occupational Hazard Survey conducted between 1972 and 1974 estimated that 1333 U.S. workers are potentially exposed to Direct Blue 6 (NIOSH, 1984); a later survey conducted by NIOSH estimated that ~800 workers are exposed (IARC, 1982a).

3.1. WATER

Pertinent monitoring data of Direct Blue 6 in water could not be located in the available literature as cited in the Appendix. For the organic dye industry in general, it has been estimated that ~90% of the dye end up on the fabric, while 10% is lost to wastewater effluents during dyeing operations (Porter, 1973; Brown et al., 1981). Losses of dyes to wastewater effluents during manufacture have been estimated to be 1-2% (Brown et al., 1981). Waste streams from dye manufacture contain dissolved inorganic salts and small amounts of dye and dye intermediate (Steadman et al., 1977). Wastewater effluents are therefore the major source of release to the aquatic environment; the efficiency of wastewater treatment operations will determine the amount of dye that reaches natural waters.

Release of dye from textile fabrics may occur from commercial or consumer washing; however, dyes in most finished products are considered to be essentially "fast" (they do not migrate or wash out) (Jones, 1979). Various after-treatments are commonly applied to fabrics dyed with direct dyes to improve the wet fastness quality (Kuehni et al., 1979).

3.2. FOOD

Pertinent monitoring data on Direct Blue 6 in food could not be located in the available literature as cited in the Appendix.

3.3. INHALATION

Direct Blue 6 has been detected in the workplace air of a textile-dyeing operation at a total airborne particulate level of 1.20-3.94 mg/m^3 (IARC, 1982a). Ambient air monitoring data could not be located in the available literature as cited in the Appendix.

Ambient atmospheric emissions may occur from ventilation of effluents at production and use sites. In addition, air effluents from production operations such as spray drying may be sources of release. Particulate emissions from these sources are usually filtered at the plant site, which should reduce significantly the amount of particulate matter actually reaching the ambient atmosphere.

3.4. DERMAL

Pertinent monitoring data regarding dermal exposure to Direct Blue 6 could not be located in the available literature as cited in the Appendix. Occupational dermal exposure has already been mentioned. The general public is exposed mainly to finished dyes after they have been applied to the product; however, there is little chance of dyes coming off in perspiration, saliva or washings if labeling instructions are followed (Jones, 1979).

3.5. SUMMARY

Occupational exposure to Direct Blue 6 has occurred (and probably still does) during its production and its use in dyeing (IARC, 1982). Wastewater effluents are probably the major source of release to the environment. Ambient monitoring data could not be located in the available literature as cited in the Appendix.

4. PHARMACOKINETICS

4.1. ABSORPTION

Pertinent data regarding the absorption of Direct Blue 6 could not be located in the available literature as cited in the Appendix. It is apparent from toxicological studies (Section 5.5.) that Direct Blue 6 is absorbed following oral administration.

4.2. DISTRIBUTION

Beaudoin and Pickering (1960) studied the distribution of Direct Blue 6 in pregnant Sherman rats. Rats were injected intraperitoneally on gestation day 8 with a dose of 14 mg/100 g (140 mg/kg) in a 2% aqueous dye solution. Rats were killed on the 20th day of gestation and maternal tissues were examined for the presence of dye. Direct Blue 6 was detected as granules in the cytoplasm of macrophage cells from the lungs, liver, uterus, ovaries, the abdominal lymph node and the kidneys. Dye was also observed in the capsule of the spleen and placenta, with the greatest accumulation found in the yolk sac and kidneys.

4.3. METABOLISM

Lowry et al. (1980) and NIOSH (1980) collected work air environment and urine samples from workers occupationally exposed to benzidine-based dyes. Levels of 3 and 7 ppb monoacetylbenzidine were detected in 2/8 workers at one plant. At another facility four workers exposed to average levels of benzidine-based dyes of 4.3, 5.2, 11.7 and 17.4 mg total particulate/m^3 had urinary concentrations of 52, 11, 10 and 112 ppb benzidine, respectively. Urine samples containing 112, 52 and 11 ppb benzidine also had concentrations of 590, 248 and 22 ppb monoacetylbenzidine present. The U.S. EPA (1980) considered these findings especially alarming because these levels of benzidine were in excess of metabolite levels found in occupationally-exposed workers with bladder cancer (Zavon et al., 1973). Samples

of urine taken from workers at other facilities where benzidine-based dyes were used contained no benzidine. Metabolite levels were well in excess of those expected for the impurities present (NIOSH, 1980). Walker (1970) suggested that benzidine-based dyes may be degraded in the gut and liver to free benzidine or monoacetylbenzidine.

Using HPLC procedures, Kennelly et al. (1984) attempted to identify DNA adducts formed after rats received an intraperitoneal injection of [ring-H^3]-Direct Blue 6. A small portion (12-14%) of the radioactivity associated with the liver DNA that was extracted with n-butanol was identified as N-(de-oxyguanosin-8-yl)-N'-acetylbenzidine. To release the remaining radioactivity, the aqueous phase and enzyme protein precipitate was treated with dithionite, a reducing agent, and then extracted with water-saturated n-butanol. HPLC analysis revealed two peaks of radioactivity, one of which was identified as N-(deoxyguanosin-8-yl)benzidine. The identity of the major water soluble DNA adduct of Direct Blue was proposed to be disodium-8-amino-2-[4(N-deoxyguanosin-8-yl)-amino biphenyl-4'-yl]azo-1-hydroxynaphthalene-3,6-disulphonate. Because benzidine was not found after dithionite reduction, it was concluded that the aqueous fraction of DNA adducts did not include unmetabolized dye nonspecifically bound to DNA. In addition, it was suggested that the water soluble adduct may result from hepatic azoreduction of Direct Blue 6 generating a species that would itself, or following o-esterifiction, be capable of reacting with DNA. This led the authors to believe that in addition to the production of benzidine, hepatic azoreduction may be an additional pathway leading to polar DNA binding species that may explain the potency of dyes such as Direct Blue 6.

Bos et al. (1984) examined the products when Direct Blue 6 was incubated with rat liver 9000 g supernatant or SPF Wistar rat hepatocytes. They found that the incubation of Direct Blue 6 with rat liver 9000 g supernatant

resulted in the appearance of a small amount of benzidine whereas significant amounts were produced when the dyes Direct Black 38 and Direct Brown 95 were subjected to the same treatment. The production of benzidine was not enhanced by pretreatment of rats with phenobarbitol. Incubation of Direct Blue 6 with rat hepatocytes resulted in the appearance of diacetylbenzidine.

In a 13-week toxicity study, NCI (1978) found benzidine and monoacetyl benzidine in urine collected over a 24-hour period from male and female B6C3F1 mice and Fischer 344 rats that had been fed Direct Blue 6 in their diets at levels up to 12,500 and 3000 ppm, respectively.

Rhesus monkeys, fed two different doses of Direct Blue 6 equivalent to 8 and 40 mg of benzidine by stomach tube, excreted 0.07 and 0.64 mg of benzidine plus monoacetyl benzidine in the urine (Rinde and Troll, 1975). These quantities represented 0.9 and 1.6% of the benzidine moiety in Direct Blue 6.

4.4. EXCRETION

Data regarding the amount and rate of excretion of Direct Blue 6 could not be located in the available literature, but the metabolites benzidine and monoacetyl benzidine were found in the urine following occupational exposure of humans to benzidine-based dyes (NIOSH, 1980) and oral exposure of rats, mice and monkeys (NCI, 1978; Rinde and Troll, 1975).

4.5. SUMMARY

Data regarding the amount or rate of absorption of Direct Blue 6 were not available. The distribution of Direct Blue 6 as granules in the cytoplasm of macrophage cells from the lungs, liver, uterus, ovaries, abdominal lymph nodes and kidneys was found following intraperitoneal injection in pregnant rats (Beaudoin and Pickering, 1960).

Kennelly et al. (1984) identified N-(de-oxyguanosin-8-yl)-N'-acetyl-benzidine and N-(deoxyguanosin-8-yl) benzidine as DNA adducts in the liver of rats injected intraperitoneally with ^{3}H-ring-Direct Blue 6. Direct Blue 6 is metobilized to benzidine and acetylbenzidine. In addition to benzidine, Direct Blue 6 may undergo hepatic azoreduction producing a species which may react with DNA. In vitro experiments with S-9 and isolated rat hepatocytes indicated that the production of benzidine is catalyzed by microsomal enzymes, while the subsequent acetylation of benzidine is catalyzed by cytosolic enzymes (Bos et al., 1984). Benzidine and monoacetylbenzidine have been detected in the urine of workers exposed to benzidine-based dyes (NIOSH, 1980) and rats, mice and monkeys exposed orally to Direct Blue 6 (NCI, 1979; Rinde and Troll, 1975).

5. EFFECTS

5.1. CARCINOGENICITY

5.1.1. Animal Studies. NCI (1978) fed groups of 10 male and 10 female Fischer 344 rats and 10 male and 10 female B6C3F1 mice Direct Blue 6 (~60% pure with no detectable benzidine contamination; detection limit-0.004% by HPLC) in corn oil in the diet for 91 days; the rats and mice were observed for 2 additional days. Rats were exposed to concentrations of 190, 375, 750, 1500 and 3000 ppm. Mice were given concentrations of 750, 1500, 3000, 6000 and 12,500 ppm. Control groups of 10 rats and mice of each sex were given the same amount of corn oil in the diet. A dose-related increase in mortality was observed in rats but not in mice. All rats of both sexes given 3000 ppm of Direct Blue 6 and one male given 1500 ppm died before the end of the study. Mortality was unaffected in the other dosage groups and in controls. Survival was unaffected in mice except for one male that died of a bacterial infection. All animals that died, were killed in a moribund condition or were killed at the end of the study were necropsied. Microscopic examination of skin, lungs, bone marrow, spleen, mandibular and mesenteric lymph nodes, thymus, heart, salivary gland, liver, bile duct (mice), pancreas, stomach, small intestine, colon, kidney, bladder, adrenals, thyroids, testes and epididymis was performed only on controls, rats treated at 1, 500 and 3000 ppm and mice treated at 12,500 ppm, unless precluded by cannibalism or autolysis. Microscopic examination of all other groups of rats was limited to the liver. Microscopic examination of other groups of mice was limited to the liver of mice treated at 6000 ppm and the spleen of mice treated at $\geq$1500 ppm. Liver tumors were observed in male rats at 1500 ppm and in female rats at 3000 ppm (Table 5-1). Liver tumors were not observed in controls or at lower dietary concentrations. The time

TABLE 5-1

Incidence of Liver Tumors in Fischer 344 Rats Given 190-3000 ppm of Direct Blue 6 (No Benzidine Contamination) in the Diet for 91 Days and Observed for an Additional 2 Days[a]

Concentration (ppm)	Sex	Tumor Incidence (p value)[b]
0	M	0/10
190	M	0/10
375	M	0/10
750	M	0/10
1500	M	8/10 (p<0.001)
3000	M	1/9
0	F	0/10
190	F	0/10
375	F	0/10
750	F	0/10
1500	F	0/10
3000	F	7/9 (p=0.001)

Quality of Evidence

Strengths of Study: Both sexes of two animal species (rats and mice) were tested by a relevant route of administration (dietary) at several concentrations. The MTD was estimated by pretesting based on body weight changes (Section 5.6.), and data were statistically analyzed. Histopathological examination was comprehensive at higher doses and limited at lower doses.

Weaknesses of Study: The test did not last for the lifespan of the organisms tested. If tested for a longer period of time, tumors may have developed at lower doses in rats or mice.

Overall Adequacy: Sufficient

[a]Source: NCI, 1978

[b]In males 2/10 liver tumors were hepatocellular carcinomas and 6/10 were neoplastic nodules. In females, 4/9 liver tumors were hepatocarcinomas and 3/9 were neoplastic nodules.

to observation of first tumor was 4-5 weeks as compared with ~78 weeks in historic controls. The lower incidence of liver tumors in males at the highest dose may have been attributable to the early mortality of these rats. A statistically significant increased incidence in preneoplastic hepatic lesions (basophilic foci) was observed in 4/9 male ($p=0.033$) and 7/9 female ($p=0.001$) rats given 3000 ppm Direct Blue 6. At 1500 ppm a lower incidence of basophilic foci (1/10) was observed in males. A significantly increased incidence of hepatic lesions occurred in additional dosage groups when foci of cellular alteration, which were possible preneoplastic lesions, were added to the basophilic foci. The incidences of basophilic foci or foci of cellular alteration were 10/10 males at 750 ppm ($p<0.001$), 8/10 males at 1500 ppm ($p<0.001$), 4/9 males at 3000 ppm ($p=0.033$), 9/10 females at 750 ppm ($p<0.001$), 10/10 females at 1500 ppm (<0.001) and 7/9 females at 3000 ppm ($p=0.001$). These preneoplastic lesions were not observed in controls or in lower dose groups. NCI (1978) concluded that Direct Blue 6 was carcinogenic to male and female rats, inducing hepatocellular carcinomas, neoplastic nodules and preneoplastic lesions within 4-13 weeks of dietary exposure. No tumors were observed in mice; however, this may be a consequence of short study duration.

Niitsu (1973) gave 20 female Wistar rats 400 mg/ℓ Direct Blue 6 (purity not specified) in drinking water for 14 months. Twelve rats were still alive at 12 months and one of these had a glandular tumor of the outer ear. No controls were maintained in this experiment.

Direct Blue 6 gave negative results in the mouse Strain A pulmonary tumor bioassay by the intraperitoneal route of administration (Maronpot et al., 1983). A high frequency of false negatives (78%) were observed for the aromatic amines in the test system. As cited in IARC (1982a), Niitsu (1973)

implanted wax pellets containing 0 or 10% Direct Blue 6 into the bladders of groups of 50 female dd mice and found that 3/21 surviving treated mice had bladder carcinomas 40 weeks after implantation as compared with an incidence of 1/36 in controls.

5.1.2. Human Studies. No studies for Direct Blue 6 alone were available. IARC (1982b,c) summarized the numerous case studies reporting the occurrence of bladder tumors in benzidine-based dyestuff workers as well as several epidemiological studies suggesting excess mortality from bladder cancer in workers occupationally exposed to benzidine-based dyes. Interpretation of these studies are confounded because workers exposed in occupational settings were exposed to mixtures of several dyes. IARC (1982b) reviewed and evaluated a Japanese hospital-based case-control study of 200 male cases of bladder cancer and 148 male controls of the same age with urinary disorders (Yoshida and Miyakawa, 1973; Yoshida et al., 1971). Seventeen (8.5%) of the cases and two (1.4%) of the controls worked in the silk-dyeing industry. Employment in the silk-dyeing industry had a relative risk of 6.8 ($p=0.002$) for bladder cancer. Seven of the 17 bladder cancer cases were kimono painters who held brushes and spatulas in their mouths while painting, thus ingesting the dyes. The dyes commonly used by the painters were benzidine-based compounds. IARC (1982a,b) concluded that exposures to benzidine-based dyes were strongly associated with the occurrence of bladder cancer in humans. Although the epidemiological data alone were inadequate to evaluate the possible carcinogenic effect to man of Direct Blue 6, when considered together with data demonstrating that benzidine was present in the urine of occupationally-exposed workers, the data provided "sufficient evidence that occupational exposure to benzidine-based dyes represent a carcinogenic risk to man" (IARC, 1982d).

5.2. MUTAGENICITY

Direct Blue 6 has been shown to be mutagenic in the frameshift sensitive Salmonella typhimurium strains TA98 and TA1538 after incubation with a metabolizing system capable of azoreduction (Table 5-2) (Prival et al., 1984; Reid et al., 1984). Prival et al. (1984) tested Direct Blue 6 in a modified Ames test protocol using the reducing agent flavin mononucleotide (FMN) and hamster liver S9 mix and found it to be mutagenic tester strain TA98 in a dose-related manner. In the absence of a reduction system (i.e., FMN and cofactors), Direct Blue 6 did not produce a dose-related mutagenic response. These results provide evidence that the azoreduction of Direct Blue 6 is one important step in its activation to a mutagen. The mutagenic intermediate of Direct Blue 6 would be the carcinogen benzidine. Prival et al. (1984) also found that hamster liver S9 was more effective than rat liver S9 in activating Direct Blue 6 to a mutagen. In fact, rat liver S9 interferes with the mutagenic action of Direct Blue 6 as it does with benzidine itself. Reid et al. (1984) also demonstrated that the mutagenic activity of Direct Blue 6 required the presence of a reduction system. Reid et al. (1984) found that Direct Blue 6 was mutagenic in Salmonella TA1538 after incubation in a bacterial reduction system (i.e., rat cecal bacteria) or with the reducing agent FMN.

Benzidine, the mutagenic/carcinogenic reduction product of Direct Blue 6, has been reported to be mutagenic to Salmonella typhimurium strains TA1538 and TA98 (IARC, 1982). It has also been shown to induce unscheduled DNA synthesis and DNA strand breaks in mammalian cell lines (IARC, 1982). Grady et al. (1986) found the induction sister chromatid exchanges by benzidine in rat and human hepatoma cell lines.

TABLE 5-2

Mutagenicity Testing of Direct Blue 6

Assay	Indicator Organism	Purity	Application	Concentration or Dose (μmol/plate)	Activating System	Response	Comments	Reference
Reverse mutation	Salmonella typhimurium TA98	NR	plate incorporation	0.1-0.3	+S-9, ±FMN[a]	±	FMN greatly increased mutagenic activity; S-9 from Aroclor-induced hamsters	Prival et al., 1984
Reverse mutation	S. typhimurium TA1538	NR	plate incorporation	0.25-1.0	+ S-9 rat, ± bacterial reduction	±	Bacterial reduction enhanced mutagenic activity	Reid et al., 1984
					+ S-9 hamster, +FMN	+		

NR = Not reported

5.3. TERATOGENICITY

Pertinent data regarding the teratogenic effects of Direct Blue 6 after oral or inhalation exposure could not be located in the available literature as cited in the Appendix.

Direct Blue 6 has been shown to cause teratogenic effects after subcutaneous and intraperitoneal injections. In one study, pregnant Wistar rats were injected subcutaneously on gestation day 8.5 with a 1% aqueous solution of Direct Blue 6 (90% pure) (Lloyd and Beck, 1966; Lloyd et al., 1965). Four groups of 7-14 dams each were injected with 50, 100, 150 or 200 mg/kg. No indication of control group data was provided. Dams were killed on day 20.5; uteri were removed and the number of resorption sites and live fetuses were counted. Live fetuses were then examined for external malformations. Results showed a dose-related increase in the number of resorptions with 100% of 53 implantations being resorbed at the 200 mg/kg dose level. A dose-related increase in the number of abnormal fetuses was also observed, with 22% abnormal fetuses at 150 mg/kg. The types of anomalies found were unspecified. Maternal lethality was observed at 100 mg/kg.

Kernis (1971) also studied the teratogenic effects of a subcutaneous dose of Direct Blue 6 ("purified"). Twenty Long-Evans black-hooded rats were injected subcutaneously with 167 mg/kg in a 1.8% aqueous dye solution on gestation day 8. Twenty-nine control rats were left untreated. On gestation day 20, rats were killed, uteri removed and the numbers of resorbed and live fetuses were counted. Results showed 20 resorbed fetuses of 247 implantation sites in the dye-treated group compared with 3/337 in the control group. There were 5 malformed and 222 normal fetuses in the treated group and 0 malformed and 354 normal fetuses in the control group. Abnormalities consisted of anophthalmia, microphthalmia, exencephaly and menigocoele. Maternal toxicity was not reported.

A study by Beaudoin (1968) showed Direct Blue 6 to be teratogenic in Wistar rats after intraperitoneal injection. Groups of 12-22 rats were injected intraperitoneally with a 2% aqueous dye solution on day 8 of gestation at doses of 7, 14 or 20 mg/100 g (70, 140 or 200 mg/kg). At the same time, 5 control rats received injections of distilled water and 10 controls were left untreated. Dams were killed on day 20, uteri were removed and the number of resorption sites and live fetuses were counted. Fetuses were fixed in Bouin's fluid or 95% alcohol for later examination. Results showed a dose-related increase in the percentage of resorbed fetuses at all doses. A dose-related increase in the percentage of malformed survivors was also observed at all doses. The major anomalies found were anophthalmia and hydrocephalus. Fetal weights were significantly reduced ($p=0.01$) in the 200 mg/kg dose group as compared with controls. Exposure to 140 and 200 mg/kg was lethal to 4/21 and 3/22 dams, respectively. Ten dams receiving an unspecified dose of Direct Blue 6 showed a decrease in weight gain, which was most significant during gestation days 8-13.

5.4. OTHER REPRODUCTIVE EFFECTS

Pertinent data regarding the reproductive effects of Direct Blue 6 could not be located in the available literature as cited in the Appendix.

5.5. CHRONIC AND SUBCHRONIC TOXICITY

NCI (1978) gave groups of 10 Fischer 344 rats and 10 B6C3F1 mice of each sex Direct Blue 6 in the diet for 91 days; they were observed for 2 additional days. The rats were exposed to concentrations of 190, 375, 750, 1500 and 3000 ppm. Mice were exposed to concentrations of 750, 1500, 3000, 6000 and 12,500 ppm (see Section 5.1.). Dose-related decreased mean body weight gain occurred in male and female rats treated with 1500 and 3000 ppm. All rats at 3000 ppm and one male rat at 1500 ppm died. No other deaths

occurred. The authors reported several trends in the data for liver lesions in rats, which revealed a progression of change from a normal to preneoplastic condition to neoplasia for the benzidine-based dyes in general. The rats surviving for longer periods of time had more proliferative liver changes as well as preneoplastic and neoplastic lesions than the rats that were moribund at earlier times. Livers that were more severely affected were of two types: a hyperplastic liver with multiple foci of cellular alteration and nodules, or a cirrhotic liver with nodules. The first type of liver changes were considered preneoplastic and neoplastic (see Section 5.1.). Cirrhosis (nodular regeneration and biliary hyperplasia, focal) and biliary hyperplasia that occurred along with neoplastic nodules and carcinomas can be considered nonneoplastic lesions. Biliary hyperplasia was observed in males at 750 ppm and in females at 1500 ppm. These lesions were not noted in rats exposed to lower levels. Nodular regeneration and portal fibrosis were observed in male and female rats exposed to 1500 ppm Direct Blue 6 but not at lower doses. The cirrhotic changes occurred in the longer-lived rats. Nonneoplastic lesions with more rapid onset included lymphoid depletion of the spleen and thymus, myeloid depletion of the bone marrow and varying degrees of biliary hyperplasia. These effects were noted in the first rats to die. Male and female rats exhibited high incidences of lymphoid depletion of the spleen and thymus, and myeloid depletion of the bone marrow at 3000 ppm. At 1500 ppm, 1/9, 1/8 and 1/10 males had the respective lesions. None of these lesions were found in controls. However, the only organ examined microscopically in rats at $\leq$750 ppm was the liver. High-dose male and female mice had slightly reduced body weight gains compared with controls. All mice survived until the end of the study. Comprehensive histological examination (see Section 5.1.) was performed only on

high-dose and control mice; livers were examined microscopically only at ≥6000 ppm and spleens were examined microscopically only at ≥1500 ppm. Male and female mice exposed to concentrations of 12,500 ppm exhibited hemosiderosis of the kidney and pigment deposition in the liver. No liver lesions were observed at 6000 ppm. Hemosiderosis of the spleen was observed at 1500 ppm in both sexes of mice.

5.6. OTHER RELEVANT INFORMATION

Oral LD_{50} values for Direct Blue 6 could not be located in the available literature as cited in the Appendix. In a 2-week range finding study, groups of five male and five female Fischer 344 rats and five male and five female B6C3F1 mice were treated with Direct Blue 6 in the diet at 0, 3000, 6000, 12,500, 25,000 and 50,000 ppm (NCI, 1978). Dose-related severely decreased food consumption and mean body weight gain occurred at ≥3000 ppm for rats. One male rat treated with 50,000 ppm Direct Blue 6 died; all other rats survived until sacrifice. Gross necropsy revealed thymic atrophy (caused by low food consumption), splenic enlargement and dose-related darkening of the spleen and kidneys at unspecified doses. In mice, no deaths occurred. Females but not males had dose-related decreased mean body weight gain at ≥6000 ppm. Hunched appearance and lethargy occurred at the higher concentrations (not otherwise specified). No toxicologically significant gross lesions were observed.

5.7. SUMMARY

Direct Blue 6 has been demonstrated to be a potent carcinogen in animal test systems. Rats exposed to dietary concentrations of 190, 375, 750, 1500 or 3000 ppm for only 13 weeks had significantly increased incidences of liver tumors (hepatocarcinomas and neoplastic nodules) at ≥1500 ppm (NCI, 1978). Liver tumors were not observed in controls or in lower dose groups.

The time to first tumor was 4-5 weeks in treated rats compared with 78 weeks in the historic controls, indicating that Direct Blue 6 is a remarkably potent carcinogen. No increased incidences of tumors were observed in mice at dietary levels $\geq$750 ppm (lowest level tested). Niitsu (1973) gave rats 500 mg/ℓ Direct Blue 6 in drinking water for 14 months and found that only one rat had a glandular tumor of the outer ear. No controls were maintained in this experiment.

There are numerous case reports available (IARC 1982a,b,c) suggesting that mixtures of benzidine formulated dyes (including Direct Blue 6) are carcinogenic in humans. Yoshida and Miyakawa (1973) and Yoshida et al. (1971) observed 17 cases of bladder cancer in a hospital-based case-control study of 200 workers occupationally exposed to benzidine-based dyes. An incidence of only 2/148 was observed in controls. Seven of the cases were kimono painters who held brushes and spatulas in their mouths while painting, thus ingesting the dyes. IARC (1982a,b) concluded that exposure to benzidine-based dyes is strongly associated with the occurrence of bladder cancer in humans. The epidemiological data are inadequate to demonstrate the carcinogenic potential of Direct Blue 6 alone, however, the data provide sufficient evidence that workers occupationally exposed to mixtures of benzidine-based dyes have an increased risk of cancer.

A consideration of metabolism and related factors is important in that formulations of Direct Blue 6 apparently have varying amounts of residual benzidine as a contaminant and that mammals including monkeys have been shown to metabolize Direct Blue 6 to benzidine and acetalbenzidine to some degree. The presence of benzidine in the urine of workers exposed to benzidine-based dyes together with significant incidences of bladder cancer demonstrates an important correlation.

The mutagenicity of Direct Blue 6 was assessed in the S. typhimurium reverse mutation assay. Positive results were obtained in strain TA98 and TA1538 in the presence of S-9 and FMN or cecal microflora (Prival et al., 1984; Reid et al., 1984).

No data concerning the teratogenic effects of Direct Blue 6 after oral or inhalation exposure were located; however, several positive studies by the subcutaneous or intraperitoneal route were found. Dose-related increased incidences of resorptions and fetal abnormalities were observed in female rats given 50-200 mg/kg of Direct Blue 6 subcutaneously during pregnancy (Lloyd and beck, 1966; Kernis, 1971). A limitation of the Lloyd and Beck (1966) study was that no controls were maintained. Controls were maintained in Kernis (1971); however, no maternal toxicity data were reported. Beaudoin (1968) injected pregnant rats intraperitoneally with 0, 70, 140 or 200 mg/kg Direct Blue 6 and observed dose-related increased resorptions and malformed fetuses (anophthalmia, hydrocephalus at all doses); however, maternal lethality was observed at the two highest doses.

Pertinent data regarding the reproductive effects of Direct Blue 6 could not be located in the available literature as cited in the Appendix.

In the 13-week dietary carcinogenicity study by NCI (1978), dose-related decreased mean body weight gain of rats occurred at $\geq$1500 ppm. All 3000 ppm rats and 1/10 males at 1500 ppm died. Treatment-related nonneoplastic lesions in rats included biliary hyperplasia in males at $\geq$750 ppm, nodular regeneration and portal fibrosis in males and females at 1500 ppm, and lymphoid depletion of the thymus and spleen and meyloid depletion of the bone marrow at 3000 ppm. None of these lesions occurred in controls. Mice treated with 12,500 ppm (highest dose) had only slightly reduced body weight

gain, and all mice at ≥750 ppm (lowest dose) survived. Nonneoplastic lesions were hemosiderosis of kidney and pigment deposition in the liver at 12,500 ppm and hemosiderosis of the spleen at ≥1500 ppm.

6. AQUATIC TOXICITY

6.1. ACUTE

Little et al. (1974) reported that the 96-hour LC_{50} of Direct Blue 6 to fathead minnows, Pimephales promelas, was >180 mg/ℓ. This concentration greatly exceeds the concentrations necessary to produce visible color (0.4-1.3 mg/ℓ).

6.2. CHRONIC

Pertinent data regarding chronic toxicity of Direct Blue 6 to aquatic organisms could not be located in the available literature as cited in the Appendix.

6.3. PLANTS

Pertinent data regarding effects of Direct Blue 6 on aquatic plants could not be located in the available literature as cited in the Appendix.

6.4. RESIDUES

Pertinent data regarding Direct Blue 6 residues in aquatic biota could not be located in the available literature as cited in the Appendix.

6.5. SUMMARY

Little et al. (1974) reported that the 96-hour LC_{50} of Direct Blue 6 to fathead minnows, Pimephales promelas, was >180 mg/ℓ. No other information concerning aquatic toxicity of this compound was available.

7. EXISTING GUIDELINES AND STANDARDS

7.1. HUMAN

NIOSH considered the benzidine-based dyes as potential human carcinogens (NIOSH, 1980, 1984) and OSHA recommended that worker exposure be reduced to the lowest feasible level (OSHA/NIOSH, 1980). In response to the Inter-agency Testing Committee recommendations, the U.S. EPA decided not to require additional testing of benzidine, o-tolidine and o-dianisidine-based dyes for health and environmental effects (U.S. EPA, 1981).

7.2. AQUATIC

Guidelines and standards for the protection of aquatic biota from the effects of Direct Blue 6 could not be located in the available literature as cited in the Appendix.

8. RISK ASSESSMENT

Direct Blue 6 has been demonstrated to be a potent animal carcinogen. Rats exposed to concentrations of 190, 375, 750, 1500 or 3000 ppm for only 13 weeks had significantly increased incidences of liver tumors (hepatocarcinomas and neoplastic nodules) at $\geq$1500 ppm (NCI, 1978). Liver tumors were not observed in controls or at lower doses. The time to first tumor was 4-5 weeks in treated rats compared with 78 weeks in the historic controls, indicating that Direct Blue 6 is a remarkably potent carcinogen. No increased incidences of tumors were observed in mice at dietary levels $\geq$750 ppm (lowest dose tested). Niitsu (1973) gave rats 500 mg/ℓ Direct Blue 6 in drinking water for 14 months and found that only one rat had a glandular tumor of the outer ear. No controls were maintained in this experiment.

There are numerous case reports available (IARC 1982a,b,c) suggesting that benzidine-based dyes (Direct Blue 6 being one of these) are carcinogenic in humans. Yoshida and Miyakawa (1973) and Yoshida et al. (1971) observed 17 cases of bladder cancer in a hospital-based case-control study of 200 (8.5%) workers occupationally exposed to benzidine-based dyes. An incidence of only 2/148 (1.4%) was observed in controls. Seven of the cases were kimono painters who held brushes and spatulas in their mouths while painting, thus ingesting the dyes. IARC (1982a,b) concluded that exposure to benzidine-based dyes was strongly associated with the occurrence of bladder cancer in humans. While the epidemiological data alone are inadequate to isolate the carcinogenic activity of Direct Blue 6, the data provided sufficient qualitative evidence that workers occupationally exposed to benzidine-based dyes have an increased risk for cancer.

A consideration of metabolism and related factors is important in that formulations of Direct Blue 6 apparently have varying amounts of residual benzidine as a contaminant and that mammals including monkeys have been shown to metabolize Direct Blue 6 to benzidine and acetalbenzidine to some degree. The presence of benzidine in the urine of workers exposed to benzidine-based dyes together with significant incidences of bladder cancer demonstrates an important correlation.

The animal evidence is limited, there being liver tumors induced in rats exposed by dietary administration but not in mice. Other experiments show that Direct Blue 6 is metabolized in part to benzidine and acetalbenzidine, albeit a small amount. By other carcinogen assessments benzidine is an EPA Group A compound, a known human carcinogen. The animal evidence together with knowledge that some Direct Blue 6 is metabolized to benzidine, and the fact that Direct Blue 6 formulations undoubtedly contain residual amounts of benzidine, elevate the weight of evidence for Direct Blue 6 to a Group A categorization.

In a collaborative manner, a consideration of the available epidemiologic data for benzidine-based dyes, with the clear findings of human bladder cancer response, together with presence of benzidine in the urine of exposed individuals, is supportive of a Group A ranking for benzidine-based dyes of which Direct Blue 6 is one.

The mutagenicity of Direct Blue 6 was assessed in the S. typhimurium reverse mutation assay. Positive results were obtained in strain TA98 and TA1538 in the presence of S-9 and FMN or cecal microflora (Prival et al., 1984; Reid et al., 1984). This evidence provides additional evidence supporting the conclusion that Direct Blue 6 may be a potential carcinogen.

No data concerning the teratogenic effects of Direct Blue 6 after oral or inhalation exposure were located; however, several positive studies by the subcutaneous or intraperitoneal route were found. Dose-related increased incidences of resorptions and fetal abnormalities were observed in female rats given 50-200 mg/kg of Direct Blue 6 subcutaneously during pregnancy (Lloyd and beck, 1966; Kernis, 1971). A limitation of the Lloyd and Beck (1966) study was that no controls were maintained. Controls were maintained in Kernis (1971); however, no maternal toxicity data were reported. Beaudoin (1968) injected pregnant rats intraperitoneally with 0, 70, 140 or 200 mg/kg Direct Blue 6 and observed dose-related increased resorptions and malformed fetuses (anophthalmia, hydrocephalus) at all doses; however, maternal lethality was observed at the two highest doses.

Pertinent data regarding the reproductive effects of Direct Blue 6 could not be located in the available literature as cited in the Appendix.

In the 13-week dietary carcinogenicity study by NCI (1978), dose-related decreased mean body weight gain of rats occurred at $\geq$1500 ppm. All 3000 ppm rats and 1/10 males at 1500 ppm died. Treatment-related nonneoplastic lesions in rats included biliary hyperplasia at 750 ppm, modular regeneration and portal fibrosis at 1500 ppm, and lymphoid depletion of the thymus and spleen and myeloid depletion of the bone marrow at 3000 ppm. None of these lesions occurred in controls. Mice treated with 12,500 ppm (highest dose) had only slightly reduced body weight gain and all mice at $\geq$750 ppm (lowest dose) survived. Nonneoplastic lesions were hemosiderosis of the kidney and pigment deposition in the liver at 12,500 ppm and hemosiderosis of the spleen at $\geq$1500 ppm.

Direct Blue 6 is considered by this assessment to be a human carcinogen and the animal data provide a basis for deriving a q_1^*. The available epidemiological studies described in Section 5.1.2. are not adequate for quantitative assessment of Direct Blue 6 because that exposure was to mixtures of dyes. The NCI (1978) study provides adequate data in rats to support a quantitative risk assessment. In that study, liver tumors (hepatocarcinomas and neoplastic nodules) were observed in 8/10 males (80%; $p<0.001$) at 1500 ppm and in 7/9 (77%; $p=0.001$) females at 3000 ppm. Liver tumors were not observed in controls. The time to first tumor was 4-5 weeks in treated rats as compared with ~78 weeks in the historic controls, indicating that Direct Blue 6 is a remarkably potent carcinogen. Using the multistage model (Howe and Crump, 1982), the q_1^* for male rats of 2.28×10^{-3} $(mg/kg/day)^{-1}$ is obtained (Table 8-1). The q_1^* is based on the incidences of tumors at 0-1500 ppm because the goodness of fit was not adequate using the high dose. The low incidence at the high dose reflects the early mortality of this group of rats. Multiplying the animal q_1^* by the cube of the ratio of the length of the animal lifespan to the length of the experiment $(L/Le)^3$ and by the cube root of the ratio of the human body weight to the animal body weight yields a human q_1^* of 8.1 $(mg/kg/day)^{-1}$. The concentrations in water associated with increased lifetime risk at levels of 10^{-5}, 10^{-6} and 10^{-7} are 4.3×10^{-5}, 4.3×10^{-6} and 4.3×10^{-7} mg/ℓ assuming that a 70 kg man consumes 2 ℓ water/day.

TABLE 8-1

Derivation of a q_1^* for Direct Blue 6

Reference: NCI, 1978

Species/strain/sex: rats/Fischer 344/male

Route/vehicle: oral/diet

Length of exposure (le) = 91 days

Length of experiment (Le) = 93 days

Lifespan of animal (L) = 730 days
Body weight = 0.175 kg (measured)

Tumor type and site: liver tumors (2/10 hepatocarcinoma; 6/10 neoplastic nodules)

Experimental Doses or Exposure (ppm)	Transformed Dose (mg/kg/day)	Incidence No. Responding/No. Tested or Examined
0	0	0/10
190	9.3	0/10
375	18.3	0/10
750	36.7	0/10
1500	73.4	8/10
3000	146.8	1/9

Transformed dose obtained by multiplying ppm x a food factor of 0.05 x (le/Le).

Unadjusted q_1^* from study = 2.2769966×10^{-3} $(mg/kg/day)^{-1}$

Human q_1^* = 8.1140084 $(mg/kg/day)^{-1}$

Note: Goodness of fit not adequate; therefore, high dose was dropped from the derivation of q_1^*

9. REPORTABLE QUANTITIES

9.1 REPORTABLE QUANTITY (RQ) RANKING BASED ON CHRONIC TOXICITY

NCI (1978) adequately described the subchronic toxicity of Direct Blue 6 (see Section 5.5.). The data provided were sufficient to determine human MEDs, CSs and RQs. Relevant information such as animal exposure levels, transformed doses and toxic effects are summarized in Table 9-1.

As seen from Table 9-1, the effects of subchronic oral exposure to Direct Blue 6 include decreased body weight gain, cirrhotic liver alterations, lymphoid depletion of the spleen and thymus and myeloid depletion of the bone marrow, hemosiderosis of the spleen and kidney and death. Equivalent chronic human doses were calculated by multiplying the animal transformed doses by the cube root of the ratio of the animal body weight to the human body weight (70 kg). As seen from Table 9-1, cirrhotic liver alterations were observed at the lowest equivalent chronic human dose (5.3 mg/kg/day). These effects were given an RV_e of 7 (Table 9-2). Multiplying the dose of 5.3 mg/kg/day by 70 kg and dividing by an uncertainty factor of 10 to approximate chronic exposure results in an MED of 37 mg/day, which corresponds to an RV_d of 3.1. Multiplying the RV_d by the RV_e results in a CS of 21.7. Effects given an RV_e of <7 (lymphoid depletion of the spleen and thymus, myeloid depletion of the bone marrow, hemosiderosis and reduced body weight gain) occurred at higher equivalent human doses; therefore CSs need not be calculated. Increased mortality, which is given an RV_e of 10, occurred at a higher equivalent chronic human dose than the cirrhotic liver alterations; however, since the severity of this effect was greater than the cirrhotic liver alterations, a CS was calculated for this effect. Multiplying the dose of 16.9 mg/kg/day by 70 kg and dividing by an uncertainty

TABLE 9-1

Oral Toxicity Summary for Benzidine-Free Direct Blue 6 Administered by Diet (10 Animals/Group)[a]

Species/Strain	Sex	Average Weight (kg)	Dose/Exposure (ppm)	Transformed Animal Dose (mg/kg/day)	Equivalent Human Dose (mg/kg/day)	Response
Rat/ Fischer 344	M	0.20	750 for ~91 days	37.5	5.3	Cirrhotic liver alterations
Rat/ Fischer 344	M,F	0.175(M) 0.125(F)	1500 ppm for ~91 days	75	10.2(M) 9.1(F)	Reduced body weight gain; cirrhotic liver changes
Rat/ Fischer 344	M,F	0.125(M) 0.10 (F)	3000 ppm for ~91 days	150	18.2(M)	Death; lymphoid depletion of the spleen and thymus and myeloid depletion of the bone marrow; cirrhotic liver lesions
Mouse/B6C3F1	M,F	0.025(M) 0.018(F)	1500 ppm for ~91 days	195	13.8(M) 16.9(F)	Hemosiderosis of the spleen
Mouse/B6C3F1	M,F	0.022(M) 0.017(F)	12,500 ppm for ~91 days	1625	110(M) 101(F)	Hemosiderosis of kidney; pigment deposition in liver; slightly decreased body weight gain

[a]Source: NCI, 1978

[b]Estimated from body weight data provided by NCI (1978).

[c]Calculated by multiplying dietary level by a food factor of 0.05 for rats and 0.13 for mice.

[d]Equivalent chronic human doses were calculated by multiplying the transformed animal dose by the cubed root of the ratio of average animal body weight to the reference human body weight (70 kg).

TABLE 9-2

Oral Composite Scores for Direct Blue 6 in Fischer 344 Rats[a]

Animal Dose (mg/kg/day)	Chronic Human MED[b] (mg/day)	RV_d	Effect	RV_e	CS	RQ
37.5	37	3.1	cirrhotic liver alterations	7	21.7	100
150	118	2.4	increased mortality	10	24	100

[a]Source: NCI, 1978

[b]The doses were divided by an uncertainty factor of 10 to approximate chronic exposure.

factor of 10 to approximate chronic exposure results in an MED of 118 mg/day, which corresponds to an RV_d of 2.4. Multiplying the RV_d by the RV_e yields a CS of 24.

As seen from Table 9-2, both CSs correspond to RQs of 100, but the higher CS for increased mortality is recommended as the basis of the RQ (Table 9-3).

9.2. WEIGHT OF EVIDENCE AND POTENCY FACTOR ($F=1/ED_{10}$) FOR CARCINOGENICITY

The carcinogenicity of Direct Blue 6 was discussed in Section 5.1. In an NCI (1978) bioassay, rats and mice were maintained on diets containing Direct Blue 6 for 13 weeks. Treated male and female rats developed liver tumors beginning at weeks 4-5 of treatment compared with a mean time to first liver tumor of 78 weeks in historic controls. Therefore, Direct Blue 6 was a potent carcinogen in rats. The incidences of hepatocellular carcinoma and neoplastic nodules are presented in Table 5-1. No evidence of carcinogenity was found in mice.

A group of 20 female rats was maintained on drinking water containing 400 mg/ℓ Direct Blue 6 (Niitsu, 1973). One of 12 surviving rats had a glandular tumor of the outer ear at 12 months. Other details were not available; therefore, this study is inadequate to assess the carcinogenicity of Direct Blue 6.

Direct Blue 6 gave negative results in the mouse strain A preliminary adenoma biossay, but there was a high rate of false negatives (78%) for aromatic amines in this study (Maronpot et al., 1983).

Implantation of wax pellets containing Direct Blue 6 into the bladders of 50 mice resulted in bladder carcinomas in 3/21 survivors at 40 weeks compared with 1/36 controls (Niitsu, 1973).

TABLE 9-3

Direct Blue 6

Minimum Effective Dose (MED) and Reportable Quantity (RQ)

Route:	oral
Dose*:	118 mg/day
Effect:	increased mortality
Reference:	NCI (1978)
RV_d:	2.4
RV_e:	10
Composite Score:	24
RQ:	100

*Equivalent human dose

IARC (1982a,d) concluded that the NCI (1978) bioassay in which hepatocellular carcinomas and neoplastic nodules were induced early in rats presented sufficient evidence that commercial Direct Blue 6 is carcinogenic to rats.

Numerous case reports of bladder cancer in dyestuff workers were reviewed by IARC (1982b,c).

A hospital-based case-control study of 200 cases of bladder cancer revealed that 17 cases worked in the silk-dyeing industry (Yoshida and Miyakawa, 1973; Yoshida et al., 1971). In 148 controls with urinary disorders other than bladder cancer, only 2 cases worked in the silk-dyeing industry. The relative risk for the silk-dyeing industry was 6.8 ($p=0.002$). The case reports and epidemiological data, considered together with evidence that benzidine and monacetylbenzidine was detected in the urine of workers occupationally exposed to benzidine-based dyes (Lowry et al., 1980), leads to the conclusion that there is sufficient evidence that exposure to benzidine-based dyes represents a carcinogenic risk to man (IARC, 1982a,b). This conclusion is supported by several authorities who have considered benzidine-based dyes to be potential human carcinogens (OSHA/NIOSH, 1980; NIOSH, 1980, 1984). IARC (1982d), however, concluded that there was sufficient evidence that benzidine-based dyes were human carcinogens but classified Direct Blue 6 as a Group 2B chemical, that is, a probable human carcinogen. In the available epidemiological studies, workers were exposed to dye mixtures and estimated levels of exposure were not specified; therefore, they cannot be used in quantitative risk assessment. The NCI (1978) study provided adequate data in rats to support a quantitative risk assessment. In that study, liver tumors (hepatocarcinomas and neoplastic nodules) were observed in 8/10 males (80%; $p<0.001$) at 1500 ppm and in 7/9 (77%; $p=0.001$) females at 3000 ppm. Liver tumors were not observed in controls.

The time to first tumor was 4-5 weeks in treated rats as compared with ~78 weeks in the historic controls, indicating that Direct Blue 6 is a remarkably potent carcinogen. The unadjusted $1/ED_{10}$ was calculated using the linearized multistage model (Howe and Crump, 1982) (Table 9-4). The $1/ED_{10}$ is based on the incidence of tumors at 0-1500 ppm because the goodness of fit was not adequate when the high dose was included. The low incidence at the high dose reflects the early mortality of this group of rats. The potency factor of 92 $(mg/kg/day)^{-1}$ was obtained by multiplying the unadjusted $1/ED_{10}$ of 2.59×10^{-2} $(mg/kg/day)^{-1}$ by the cube of the ratio of the lifespan of rats to the duration of the study and by the cube root of the ratio of human body weight to the rat body weight.

A consideration of metabolism and related factors is important in that formulations of Direct Blue 6 apparently have varying amounts of residual benzidine as a contaminant and that mammals including monkeys have been shown to metabolize Direct Blue 6 to benzidine and acetalbenzidine to some degree. The presence of benzidine in the urine of workers exposed to benzidine-based dyes together with significant incidences of bladder cancer demonstrates an important correlation.

Limited animal evidence has shown liver tumors induced in rats exposed by dietary administration. No tumors were induced in mice. Other experiments have shown that Direct Blue 6 is metabolized to some extent to benzidine and acetalbenzidine. By other carcinogen assessments benzidine is an EPA Group A compound, a known human carcinogen (U.S. EPA, 1986b). The animal evidence together with knowledge that some Direct Blue 6 is metabolized to benzidine together with the knowedge that Direct Blue 6 formulations undoubtedly contain amounts residual of benzidine elevate the weight of evidence for Direct Blue 6 to a Group A categorization.

TABLE 9-4

Derivation of Potency Factor (F) for Direct Blue 6

Reference:	NCI (1978)
Exposure Route:	oral
Species:	rat
Strain:	Fischer-344
Sex:	male
Vehicle or Physical State:	dietary
Body Weight:	0.175 kg (measured)
Duration of Treatment:	91 days
Duration of Study:	93 days
Lifespan of Animal	730 days
Target Organ:	liver
Tumor Type:	hepatocellular carcinoma and neoplastic nodules
Experimental Doses/Exposure (ppm):	0, 190, 750, 1500, 3000
Transformed Doses (mg/kg/day)*:	0, 9.3, 18.3, 36.7, 73.4, 146.8
Tumor Incidence:	0/10, 0/10, 0/10, 0/10, 8/10, 1/9
Unadjusted $1/ED_{10}$:	2.594828×10^{-2} $(mg/kg/day)^{-1}$
$1/ED_{10}$ (F Factor):	92 $(mg/kg/day)^{-1}$

*Calculated by multiplying the concentration in the diet by 0.05 and by duration of treatment/duration of the study.

Note: Goodness of fit not adequate; therefore, high dose was dropped from the derivation of $1/ED_{10}$

In a collaborative manner, a consideration of the available epidemiologic data for benzidine-based dyes with the clear findings of human bladder cancer response together with presence of benzidine in the urine of exposed individuals is supportive of a Group A ranking for benzidine-based dyes of which Direct Blue 6 is one. An F factor of 92 $(mg/kg/day)^{-1}$ places Direct Blue in potency Group 2 which, along with the A classification, gives Direct Blue a HIGH hazard ranking under CERCLA.

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APPENDIX

LITERATURE SEARCHED

This profile is based on data identified by computerized literature searches of the following:

GLOBAL
TSCATS
CASR online (U.S. EPA Chemical Activities Status Report)
CAS online STN International
TOXLINE
TOXBACK 76
TOXBACK 65
RTECS
OHM TADS
STORET
SRC Environmental Fate Data Bases
SANSS
AQUIRE
TSCAPP
NTIS
Federal Register

These searches were conducted in October, 1985. In addition, hand searches were made of Chemical Abstracts (Collective Indices 6 and 7), and the following secondary sources were reviewed:

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